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Short communication

Automation and validation of the high-performance liquid chromatographic–radioimmunoassay method for the determination of lacidipine in plasma

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Abstract

The automation and validation of the HPLC–radioimmunoassay (RIA) method for the determination of lacidipine are reported. The solid-phase extraction step was automated by the introduction of the ASPEC system. A two-column system was adopted for the HPLC purification. The RIA was converted from heterogeneous to homogeneous by the scintillation proximity assay system and automated using an automatic dilution system. All characteristics in terms of accuracy, precision, specificity, and linearity resulted similar to the manual version. The quantification limit was set to 40 pg/ml. The new version of the method increased the number of samples assayed per month two- to three-fold.

1. Introduction

Lacidipine is a new dihydropyridine calcium antagonist possessing potent and long-lasting antihypertensive properties. The suggested therapeutic dosage is one 4-mg tablet daily; therapeutic plasma levels are very low (under 5 ng/ml at the peak time, and under 0.1 ng/ml at 24 h). Due to the low systemic levels, conventional analytical methods, such as high-performance liquid chromatography (HPLC) with ultraviolet detection (UV) are of limited use for bioanalytical determinations of lacidipine in plasma. After the initial failure of a radioimmunoassay (RIA) method development due to unknown cross-reacting substances, a highly sensitive and specific method was established by coupling a solid-phase extraction (SPE) of the plasma sample to a HPLC separation followed by RIA of the HPLC fractions [1]. However, the rather time-consuming sample work-up, the relative instability of the analyte in mobile phase and the complexity of the whole procedure allowed a maximum throughput of 300 samples assayed in one month by two analysts. Therefore, attempts were made to improve the productivity. To achieve this, the three main steps (SPE, HPLC and RIA) of the method were independently modified and automated (Fig. 1).

The manual SPE vacuum box method was replaced by the ASPEC (Gilson) system to provide fully automated SPE. Up to 60 samples were automatically processed by this system in a

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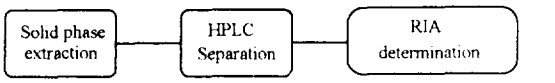

Manual Method	vacuum box	single column	charcoal separation manual
Automated Method	ASPEC	2 parallel columns	SPA automated with PROBE

Fig. 1. Characteristics of the two versions of the HPLC–RIA method for the determination of lacidipine in plasma.

single batch using the same extraction columns as for the manual procedure.

Due to the instability of the analyte in mobile phase, the 60 samples extracted, after concentration in a centrifugal evaporator, have to be further purified as quickly as possible. So a HPLC system with two columns working in parallel was adopted. The autosampler alternated the injections in the two columns so that the lacidipine peak was separated on a column and collected, while matrix-related substances eluting from the other column were sent to waste. With this system the time required for a sample analysis was halved, and a batch of 60 samples was processed in about 5 h.

In spite of the improvement achieved by the automation of SPE and HPLC steps, the RIA, based on the separation of bound and free radiolabel by the charcoal method, was still precluding a significant increase of the method productivity. A means for further optimisation emerged with the development of the scintillation proximity assay (SPA) [2,3], whereby the conventional RIA was easily switched to a one-tube procedure with the advantage of replacing manual handling by an automated pipetting system. In short, SPA is based on the use of fluoromicrospheres coated with antibodies against the primary antibody. If the labelled ligand bound to the specific antibody used in RIA binds to the secondary antibody on the surface of the fluoromicrospheres, light emission takes place and can be recorded by liquid scintillation counters.

This paper describes the validation of the automated HPLC–RIA procedure for the assay of lacidipine in human plasma and a comparison with the previous manual version of the method.

2. Experimental

2.1. Apparatus

The automatic preparation of the samples was performed by the ASPEC (Automatic Sample Preparation with Extraction Columns) supplied by Gilson (Milan, Italy). Bondelut C_{18} 500-mg cartridges were supplied by Analytichem International (Step Bio, Bologna, Italy). The HPLC apparatus consisted of two Model 305 pumps (Gilson, Milan, Italy), a Model 232bio automatic injector system equipped with two Rheodyne injection valves (Gilson), a Model 870 UV detector (Jasco, Milan, Italy), an automated Rheodyne 6-port switching valve (Gilson) and a Model 201 fraction collector (Gilson).

All separations were carried out on Hypersil ODS 3 μm, 60 × 4.6 mm analytical columns (Hewlett-Packard, Padua, Italy), and dry-packed 30 × 2 mm guard column filled with RP-8 30–40 μm (Merck-Bracco, Milan, Italy) was used. Guard and analytical columns were kept at 40°C by a TCM oven (Waters, Milan, Italy). Evaporation of solvents was performed by heating at about 40°C in centrifugal evaporator Univapo (Step Bio). All pipetting steps were done by the Probe 1000 (Camberra Packard, Milan, Italy). Radioactivity was measured with the Topcount (Camberra Packard).

2.2. Materials

Lacidipine was supplied by Pharmacy Department, Glaxo S.p.A. (Verona, Italy). The tritiated radiolabelled tracer, prepared by catalytic hydrogenation of lacidipine, was supplied by Amersham Radiochemical Centre (Amersham,

UK) at an initial specific activity of 1.81 TBq/mmol. The antiserum CM 766-3rd was the same as used for the manual version of the method. Anti-rabbit SPA reagent (code RPN140) was supplied by Amersham. All the other reagents were of analytical or HPLC grade.

2.3. Preparation of solutions

Standard solutions

Lacidipine stock solutions were prepared by dissolving the compound in methanol. Subsequent dilutions were made in acetonitrile–water (1:1). Stock solution and dilutions were stored at 4°C and were stable for at least 6 months.

Heparinised horse plasma samples (3 ml) were used every day to prepare the calibration samples by spiking with up to 200 μl standard solutions of lacidipine. The final concentrations of 0, 41, 82, 164, 328, 656, 1312, 2624 pg/ml were obtained. Spiked samples for accuracy and precision evaluation were prepared in the same way.

The equivalence between human plasma measurement and horse plasma measurement has been proved during the validation of the previous version of the method [1].

Solid-phase extraction solutions

The acid washing solution was acetonitrile–water–orthophosphoric acid 88% (10:89:1, v/v/v). The basic washing solution was acetonitrile–water–ammonia 33% (10:88:2, v/v/v).

RIA buffer

Disodium hydrogenphosphate–disodium ethylenediaminetetraacetate (Na_2EDTA) (0.05 *M*, pH 7.4) containing 0.1% of bovine serum albumin (BSA) and 0.1% sodium azide was used for RIA. The solution was stable for two weeks.

Tracer working solution

The stock solution was diluted 1:50 in ethanol (A, 0.74 MBq/ml). This diluted solution was stable up to six months when stored at −25°C. On each day of the experiment a further dilution was prepared diluting 70 μl of the dilution A with 8 ml of ethanol. The final concentration of the tracer was about 1.6 ng/ml equivalent to 6.5 kBq/ml.

Antibody working solution

The original antiserum was stored in small aliquots at −25°C. Samples of 0.5 ml were diluted 1:35 adding 16.625 ml of RIA buffer and 0.875 ml of Trasylol (Bayer, Milan, Italy) corresponding to 17500 IU of kallikrein.

On each day of the experiment this solution was appropriately diluted 1:100 in RIA buffer to bind about 50% of the tracer in the experimental condition.

SPA reagent

A 50-ml amount of the RIA buffer was added to the reagent flask and the solution was then shaken for 1 h. The reagent stored at 4°C was stable for 1 week.

2.4. Assay procedures

Solid-phase extraction (SPE)

Plasma samples (3 ml) were thawed and deproteinized adding the same volume of acetonitrile. The tubes were then vortexed and centrifuged at 1500 *g* for 10 min. The supernatant was transferred in a clean tube. The ASPEC activated the SPE cartridges with 3 ml of methanol and 2 ml of acetonitrile–water (1:1) and the sample supernatant was applied onto the cartridges. After that the instrument washed the cartridges with 1.5 ml of basic solution, 1.5 ml of acid solution and 2 ml of acetonitrile–water (1:1). Lacidipine was eluted with 2 × 1 ml of acetonitrile.

The extracts were reduced to dryness in a centrifugal evaporator and then reconstituted in 75 μl of mobile phase for the injection into the HPLC.

HPLC purification

The purification step was performed by a HPLC system with two parallel columns. A scheme of the chromatographic system, with the two switching valves, is shown in Fig. 2. The autosampler alternates the injection in the two

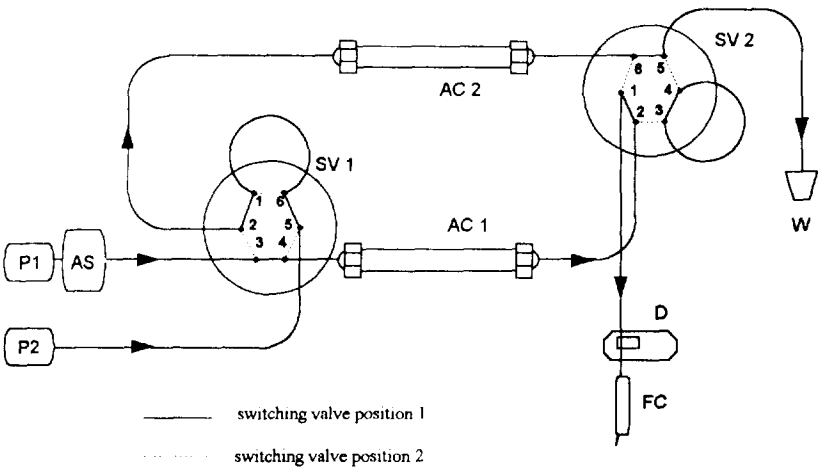

Fig. 2. Scheme of the HPLC apparatus. P1 = pump 1; P2 = pump 2; AS = autosampler; AC1 = analytical column 1; AC2 = analytical column 2; W = waste; D = detector; FC = fraction collector; SV1 = switching valve 1; SV2 = switching valve 2. When the SV1 and SV2 are in position 1, AC1 is separating the sample and AC2 is being washed, this situation is reversed when SV1 and SV2 are in position 2.

columns, halving the analysis time per sample (5 min). The samples were separated flushing the columns with acetonitrile–methanol–water (6:66:28, v/v/v) at 1 ml/min at 40°C. The detection wavelength was 300 nm. The retention time of lacidipine was about 4 min.

A fraction of 1 ml was collected in 8-ml glass tubes at the authentic retention times previously determined for lacidipine standard. Lacidipine was not stable in the mobile phase when exposed to air, so a Gilson pipette tip was placed onto the top of the collection tubes to minimise the exposure to air. In these conditions there was no loss of lacidipine for at least 2 h.

RIA determination

All pipetting steps were done by a Probe 1000 automatic diluter system. Evaporated HPLC fractions were redissolved with 100 μl of the tracer solution and 700 μl of RIA buffer and then mixed by the Probe. The samples were pipetted in two wells, 400 μl each, of a 24-well microplate (Optiplate, Camberra Packard) and 100 μl of the antibody solution and 100 μl of SPA reagent in RIA buffer were added. The microplates were shaken overnight and subsequently measured in the Topcount multichannel counter, which analyses 6 samples per time point. Counting time was 10 min.

The antibody bound fraction was expressed as percentage B/B_0 (the amount bound relative to the amount bound at 0 concentration) versus log lacidipine plasma concentration. The dose–response curve was a four-parameter logistic function. Calculations were performed using the RIASMART software (Camberra Packard).

3. Results

3.1. Method validation

Intra- and inter-assay validation

Intra-assay data were obtained from six assays at each concentration of the calibration samples carried out in a single day, whereas inter-assay data were obtained from two assays at each concentration level of the calibration samples, repeated for four different days. The results are reported in Table 1. The intra-assay precision (C.V.) ranged from 6.7% to 22.7% for the lowest concentration. The bias ranged from −12.8% to 14.6%. The theoretical nominal concentration

Table 1
Automated HPLC–RIA assay method of lacidipine in plasma: intra- and inter-assay validation

Nominal concentration (pg/ml)	Concentration found (pg/ml)					
	Mean	Confidence interval at 5% of s.l.	*n*	S.D.	C.V. (%)	Bias (%)
Intra-assay						
41	47	36–58	6	11	22.72	14.63
82	93	76–110	6	17	17.92	13.41
164	151	125–178	6	25	16.67	−7.83
328	339	315–363	6	23	6.72	3.40
656	647	564–730	6	79	12.26	−1.40
1312	1311	1193–1429	6	113	8.6	−0.06
2624	2289	1854–2724	6	415	18.11	−12.76
Inter-assay						
41	52	35–69	6	16	31.06	26.83
82	83	68–98	6	14	17.43	1.42
164	159	139–179	8	24	15.32	−3.05
328	367	319–415	8	58	15.75	11.89
656	673	578–768	8	114	16.90	2.55
1312	1401	1208–1595	8	232	16.54	6.8
2624	2529	2216–2842	8	375	14.83	−3.63

s.l. = Significance level; *n* = number of observations; S.D. = standard deviation; C.V. = coefficient of variation; bias = (concentration found − nominal concentration) × 100/nominal concentration.

was always within the 95% confidence limits of the determined means.

The inter-assay precision (C.V.) was around 15% for all the calibration levels except for the lowest concentration (31.1%). The bias ranged from −3.6% to +26.8%. Again the theoretical nominal concentration was always within the 95% confidence limits of the determined mean. Therefore the method can be considered unbiased at the 5% of significance level.

Limit of detection and limit of quantification

The limit of quantification (LOQ) was defined as the concentration of the lowest calibration standard assayed with acceptable accuracy and precision. For the manual method [1] a limit of detection of 20 pg/ml was obtained and even if poor levels of accuracy and precision were observed at this concentration, it was assumed also as LOQ. The limit of detection of 20 pg/ml was also confirmed for the automated procedures, but the low precision and accuracy suggested to set the LOQ at 40 pg/ml.

Overall recovery

To calculate the overall recovery of the method, a standard curve was prepared assaying standard solutions in ethanol. Three standard plasma samples for each calibration point were also assayed, and their real concentrations (the values obtained from the standard curve) were calculated. The mean overall recovery was the ratio between the real concentration and the nominal concentration. The overall recovery resulted 81.5% ranging from 69.2% to 98.1%.

Specificity

This procedure is based on the previously described HPLC–RIA method [1], so the data regarding the specificity of the antiserum against the known metabolites of lacidipine were considered valid also for the automated version. Adopting the same method, the specificity of the antiserum was tested against the following marketed drugs: digoxin, antipyrine, tolbutamide, warfarin, diclofenac, fosinopril and fosinoprilat. Three concentrations of each substance were

tested, the highest one being 2–10 times the typical peak concentration found in plasma after an oral administration at the therapeutic dose in man. None of these drugs displaced more than 5% of the bound tracer from the antibodies.

Throughput

The maximum batch size for the method is 60 samples, allowing the assay of 10 calibration samples, 6 quality control samples and 44 test samples in each batch. About 400 unknown samples per person per month is the estimated throughput of this method.

Comparison with the manual method

Intra-assay variability of the automated and manual versions of the method was compared using the data obtained in the respective validation studies. Concentration values were normalised to 40, 80, 160, 320, 640, 1280, 2560 to allow the comparison between the two validation studies (Table 2). Using the *F*-test, standard deviations were significantly worse for the automated procedure at the concentration level of 40 (at 5% significance) and 80 pg/ml (at 1% significance). However, accuracy of the automated procedures seems to be slightly better. The most important improvement obtained by automating the method was the increase of the number of samples that can be assayed per month.

4. Discussion

The automation of the SPE increased the batch size from 32 to 60, with a remarkable reduction of the manual handling of the samples. In addition, the use of a HPLC system with two separation columns enables the purification of all the extracted samples in a reasonable time. Nevertheless, the most relevant variation introduced was the conversion of the RIA from a heterogeneous to a homogeneous system made by scintillation proximity assay, and the consequent automation using an automatic dilution system.

The new version of the method increased the number of samples assayed per month by a factor 2 to 3. A validation study was performed to assess the performance of the new procedure and to compare this with the performance obtained with the manual method. Taking into account the complexity of the assay procedure, the precision of the assay obtained can be considered acceptable. Accuracy and precision

Table 2
Automated HPLC–RIA assay method of lacidipine in plasma: comparison with the manual method

Nominal concentration (pg/ml)	Concentration found (pg/ml)						*F*-test		
	Automated ($n = 6$)			Manual ($n = 8$)			Test value	Table value (5% s.l.)	Table value (1% s.l.)
	Mean ± c	S.D.	C.V. (%)	Mean ± c	S.D.	C.V. (%)			
40	46 ± 12	11	22.72	51 ± 3	4	8.31	6.30	5.29	9.52
80	91 ± 18	17	17.92	81 ± 3	3	4.28	22.80	5.29	9.52
160	147 ± 24	24	16.67	160 ± 9	11	6.90	4.87	5.29	9.52
320	331 ± 23	22	6.72	336 ± 23	28	8.39	1.58	6.85	14.94
640	631 ± 81	77	12.26	545 ± 68	81	14.89	1.11	6.85	14.94
1280	1279 ± 115	110	8.60	1076 ± 86	103	9.57	1.14	5.29	9.52
2560	2233 ± 425	405	18.11	2626 ± 47[a]	56	2.24	–	–	–

Mean ± c = 95% confidence interval of the mean.

[a] These samples were reconstituted with a higher volume, so they are not statistically comparable.

were also similar in the two methods. The limit of quantification (LOQ) was set at 40 pg/ml.

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